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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(c)

Atty Ref No. 038586-323; Client Ref. No. 2002-258

INVENTOR(s)

Given Name (first and middle [if any]	Family Name or Surname	Residence (City and either State or Foreign Country)
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TITLE OF THE INVENTION (280 characters max)

COMPOUNDS AND METHODS FOR ENHANCING BONE FORMATION

☐ *Additional inventors are being named on the _____ separately numbered sheets attached hereto*

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ENCLOSED APPLICATION PARTS (*CHECK ALL THAT APPLY*)

☑ Specification *Number of Pages* Cover + 9 pages

☑ Drawing(s) *Number of Sheets* Four (4) sheets (Figures 1-9)

☐ Application Data Sheet See 37 C F R. 1.76

☑ Express Mail Label No. EV089381245US

☐ CD(s), Number

☑ Other (specify)

Acknowledgment postcard

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (*check one*)

☑ Applicant claims small entity status. See 37 C.F.R. 1.27

☑ A check or money order is enclosed to cover the filing fees

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The Invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ 4 No

☑ 5 Yes, the name of the U.S Government agency and the Government contract number are: National Institutes of Health / National Institutes of Aging Grant No. P60-AG10415-11

Respectfully submitted,

SIGNATURE [signature] Date 8.29.2002

REGISTRATION NO. (if appropriate) 6 45,323

TYPED or PRINTED NAME Daphne L. Burton

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

Attorney Docket No. 038586-0323
Client Do[redacted]t No.: 2002-258

U.S. Provisional Application

Certificate of Mailing (Under 37 CFR 1.10)

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PROVISIONAL PATENT APPLICATION
UNDER 37 C.F.R. 1.53(C)

For

COMPOUNDS AND METHODS FOR ENHANCING BONE FORMATION

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COMPOUNDS AND METHODS FOR ENHANCING BONE FORMATION

[0001] This research is sponsored by National Institutes of Health/National Institutes of Aging, Grant #P60 AG10415-11.

BACKGROUND OF THE INVENTION

[0002] Osteoporotic bone loss is a major cause of morbidity and mortality in the aging population. This is a result of increased bone resorption by osteoclastic cells and decreased bone formation by osteoblastic cells with age and after menopause in women. Accumulating evidence suggests that the number and activity of osteoblastic cells decrease with age, however the reason for this change is not clear.

[0003] Recently, efforts in developing drugs to prevent and reverse osteoporosis have shifted from inhibitors of osteoclastic bone resorption to stimulators of osteoblastic bone formation. Therefore finding new anabolic agents and targets for stimulating bone formation is of utmost interest.

[0004] A set of naturally occurring, non-protein compounds have been identified that when used in combination, stimulate the osteogenic differentiation of osteoblast progenitor marrow stromal cells (also known as mesenchymal stem cells). When used on marrow stromal cell cultures, osteogenic differentiation is induced as early as 4 hours after treatment, and induces a 35-fold increase in alkaline phosphatase activity in those cells. Induction of osteogenic differentiation by these compounds is accompanied by a 40-fold increase in mineralization after 10 days of treatment. When used in mouse calvarial organ cultures, the compounds stimulate significant bone formation. Furthermore, these compounds completely inhibit adipogenic differentiation of marrow stromal cells, which is increased at the expense of osteoblast formation in osteoporotic bone. Therefore, activation of marrow stromal cells with a cocktail of these compounds stimulates bone mineral formation and is a novel and potentially important strategy for intervention with osteoporosis.

[0005] Currently, the only FDA approved anabolic agent for the treatment of osteoporosis is parathyroid hormone (PTH). Although PTH has been found to have anabolic effects when used intermittently in osteoporotic patients, the dose must be

strictly regulated since continuous treatment with PTH and/or its accumulation may have adverse systemic effects. No other bone anabolic agent is currently in use, although much research is being done in that area. Our studies indicate that the use of the compounds we identified may be another potential therapeutic intervention with bone loss. This strategy may be useful in: local stimulation of bone formation and repair, treatment of local bone destruction such as in periodontitis, periodontal regeneration, alveolar ridge augmentation for tooth implant reconstruction, treatment of non-union fractures, propagation of osteogenic cells from homologous adult mesenchymal stem cells, enhanced healing of fractures, or for systemic administration and targeting to bone in order to prevent osteoporosis.

INVENTION SUMMARY

INTRODUCTION

[0006] An important problem facing the aging population is the decrease in bone mass that results in fragile bones and increased risk of fractures. In fact, osteoporosis is a major cause of morbidity and mortality in the elderly and the annual cost to the U.S. health care system is at least ten billion dollars. Both men and women suffer from osteoporotic bone loss with age. The mechanism by which bone health is affected by osteoporosis appears to be due to decreased bone formation by osteoblasts and increased bone resorption by osteoclasts. Decrease in sex hormones with age impacts these detrimental changes.

[0007] At present, the only treatments for osteoporosis are those that target bone resorption by osteoclasts. These FDA approved therapeutics include the bisphosphonates, the selective estrogen receptor modulators, calcitonin, and vitamin D and calcium supplementation. However, these strategies only result in only small improvements in bone mass and are not sufficient for total prevention or treatment of osteoporosis.

[0008] Recently, parathyroid hormone (PTH) has been demonstrated to have anabolic effects when given intermittently to humans, thus increasing bone mass. However, although promising results have been found with PTH, there are still unresolved issues

with respect to the safety and use in humans. Therefore, improved treatments for osteoporosis are desired.

[0009] Normal bone remodeling, which occurs throughout the adult life in order to preserve the integrity of the skeleton, involves bone resorption by osteoclasts and bone formation by osteoblasts. The osteoblasts come from a pool of marrow stromal cells (also known as mesenchymal stem cells) that are pluripotent and can differentiate into osteoblasts, chondroblasts, fibroblasts, myoblasts, and adipocytes. These cells are reduced in number and in osteogenic activity with aging and in osteoporosis. Additionally, there is an increase in formation of adipocytes in osteoporotic bone marrow that appears to be at the expense of osteoblast formation. Therefore, potential strategies for intervention with osteoporosis and the effects of aging on bone are 1) to stimulate the number or activity of marrow stromal cells that become osteoblastic, and 2) to inhibit their ability to undergo adipogenic differentiation.

[0010] Oxysterols are oxygenated derivatives of cholesterol and are important in regulating cholesterol biosynthesis. These molecules have also been implicated in regulation of other physiologic and/or pathologic processes including apoptosis, atherosclerosis, necrosis, inflammation, and immunosuppression. However their possible role in bone homeostasis has not yet been described. Oxysterols bind to and activate nuclear hormone receptors called liver X receptors (LXR) which then bind to consensus binding sites on the promoter of genes that are regulated by LXR. LXR are members of the nuclear hormone receptor family and were only recently identified as intracellular receptors for oxysterol binding. To date, it is not known whether there are other orphan nuclear hormone receptors that can also serve as for oxysterol binding sites that could mediate some of the regulatory effects of oxysterols.

[0011] In the present report, we have identified specific oxysterols that when given to mouse marrow stromal cells in vitro stimulate their osteogenic activity and result in formation of bone mineral in vitro. In addition, we have found that those same osteogenic oxysterols can block adipogenesis of marrow stromal cells, and therefore not only increase their osteogenic capacity but also inhibit their differentiation into adipocytes.

[0012] This is the first report of the possible regulatory effect of oxysterols on bone cells and on pluripotent mesenchymal stem cells, and may provide a new set of molecules for treatment and/or prevention of osteoporosis.

RESULTS

[0013] 1. Oxysterols induced alkaline phosphatase activity, an early marker of osteoblastic differentiation, in marrow stromal cells (MSC) (Figure 1). We found that the activity of this enzyme, which is important for bone formation and osteoblastic differentiation, was induced several fold after only 24 hours of treatment with the oxysterols, either alone or in combination (Figure 2).

[0014] 2. The use of cytochrome P450 inhibitor SKF525A potentiated the osteogenic effects of the oxysterols, suggesting that they are metabolized and inhibited by the cytochrome P450 enzymes (Figure 3). Furthermore, treatment with stimulator of cytochrome P450 enzymes, Benzylimidazole, inhibited oxysterol effects, perhaps through enhancing their degradation (Figure 4).

[0015] 3. Treatment of MSC with combination oxysterols 22R-hydroxycholesterol and 20S-hydroxycholesterol, each at 5 μM, induced alkaline phosphatase activity after 4-96 hours of treatment and measured 4 days post-treatment (Figure 5). The effect of combination oxysterols on marrow stromal cells was dose-dependent both for the induction of alkaline phosphatase activity (Figure 6) and bone mineral formation (Figure 7).

[0016] 4. Other combinations of oxysterols that had stimulatory effects on osteogenic activity of marrow stromal cells were 22R+pregnanolone, 20S+pregnanolone, both at 5 μM. Pregnanolone is an activator of another nuclear hormone receptor called PXR. However, the most effective combination oxysterols that consistently induced robust osteogenic activity of the marrow stromal cells including both induction of alkaline phosphatase and mineral formation was 22R+20S. (The oxysterols when used alone were able to significantly induce alkaline phosphatase activity, however mineralization was only induced with combination oxysterols.) In a separate set of experiments, M2 cells were treated with 22R- and 20S-

hydroxycholesterol, each at 5 or 10 μM alone, or in combination at a concentration of 5 μM each for 4 days. The oxysterols were then removed and replaced with fresh media without oxysterols. After 19 days of treatment, 2 mM calcium chloride was added to all untreated and oxysterol treated cells. After an additional 2 days of incubation, von Kossa staining for detection of calcium phosphate mineral showed very little mineralization in the control untreated cells and cells treated with 22R-hydroxycholesterol or 10 μM, or 20S-hydroxycholesterol at 5 μM alone. However, cells treated with 10 μM 20S-hydroxycholesterol did have significantly more mineral and very robust mineralization was found in the cell cultures treated with the combination of 22R- and 20S-hydroxycholesterol.

[0017] 5. The osteogenic oxysterols inhibited adipogenesis in MSC cultures (Figure 8).

[0018] 6. LXR-beta is present in MSC, however the osteogenic effects of the oxysterols described above appear not to be through LXR since treatment with specific activators of LXR inhibited osteoblastic differentiation of those cells (Figure 9). Similar inhibitory effects of the LXR activators were seen on mineralization of marrow stromal cells.

[0019] 7. In addition to the in vitro studies described above, in preliminary studies we found that the osteogenic oxysterols induce bone formation in mouse calvarial organ cultures, a method commonly used to demonstrate osteogenic effects of compounds.

DISCUSSION

[0020] It appears that the oxysterols identified have osteogenic potential as evidenced by their ability to induce osteoblastic differentiation and mineralization of MSC in vitro. In addition, they have anti-adipogenic effects and inhibit adipocyte formation of MSC treated with a PPAR-γ activator, troglitazone. PPAR- γ activates the transcription of genes involved in adipogenesis, and therefore is called the master regulator of adipogenesis. Both the induction of osteogenic differentiation and inhibition of adipogenic differentiation would result in enhanced bone formation and therefore would be beneficial in treatment and/or prevention of osteoporosis. Bone fracture healing is

impaired in the elderly mainly due to the reduced number and activity of the MSC that would normally migrate into the fracture site and allow for new bone formation to occur. Oxysterols locally administered to the site of fracture may stimulate the osteogenic activity of osteogenic cells and therefore enhance and accelerate bone repair. In addition, oxysterols administered systemically and targeted to bone, may also be beneficial in reversing osteoporosis by increasing bone formation. If administered in combination with cytochrome P450 inhibitors, their optimal minimum effective dose may be reduced. In addition, activators of LXR causing an inhibition of osteoblastic differentiation of MSC, inhibition of this pathway may have stimulatory effects on bone formation. Overall, further studies will determine if oxysterol treatment may be an effective interventional strategy against osteoporosis.

MATERIALS AND METHODS

[0021] Cell culture - Murine marrow stromal cells, M2-1 OB4, were purchased from American Type Tissue Collection and grown in RPMI medium containing 10% fetal bovine serum (FBS). At confluence, the cells were treated in RPMI containing 5% FBS plus ascorbate at 50 µg/ml and β-glycerophosphate at 3 mM to induce osteoblastic differentiation. Adipogenic differentiation was induced by treating the cells in growth medium plus 1 0 ~M troglitazone. All oxysterols were purchased from Sigma chemical company, SKF525A was purchased from BioMol research laboratories.

[0022] Oxysterol treatment - Oxysterol treatment was performed in a variety of doses as indicated. Cells were always treated at 90% confluence. In order to assess the effects of oxysterols on osteoblastic differentiation, the cells were treated in osteogenic medium described above. In order to assess the effects of oxysterols on adipogenic differentiation, the cells were treated in adipogenic medium as described above.

[0023] Assays - Alkaline phophatase activity was measured in whole cell lysates by a colorimetric assay. Quantification of mineralization was done by measuring the amount of ^{45}Ca incorporation into extracellular matrix of the cells. Evidence for the mineral formation was found by von Kossa staining for calcium phosphate. Adipocytes were identified by oil Red 0 staining.

[0024] Applications of this new technology include:

[0025] 1. Local application to sites of bone fracture in order to enhance and accelerate the healing process.

[0026] 2. Systemic administration for prevention of osteoporotic bone loss through stimulation of osteogenesis and inhibition of adipogenesis of cells in the bone marrow, preferably by targeting of the compounds to bone.

[0027] 3. Administration to stem cells derived from adults in order to induce formation of osteoblastic cells that can be injected into the sites of bone fracture to enhance healing.

[0028] 4. Inhibition of LXR in osteoprogenitor cells in order to alleviate inhibitory effects of LXR activators that would normally inhibit bone formation.

[0029] References:

[0030] 1. Manolagas SC. Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. 2000 Endocrine Reviews 21:115-137.

[0031] 2. Lopez FJ. New approaches to the treatment of osteoporosis. 2000 Current Opinion in Chemical Biology 4:383-393.

[0032] 3. O'Connell MB. Prevention and treatment of osteoporosis in the elderly. 1999 Pharmacotherapy 19:78-208.

[0033] 4. Peet DJ, Janowski BA, Mangelsdorf DJ. The LXRs: a new class of oxysterol receptors. 1998 Current Opinion in Genetics and Development 8: 571-575.

CLAIMS

I CLAIM:

1. The method of treating osteoporosis with a selected dose of at least one compound selected from the family of oxysterols, precursors or derivatives thereof.

2. The method of claim 1 wherein the oxysterol is any one or combination of 20S-hydroxycholesterol, 22S-hydroxycholesterol and/or 25-hydroxycholesterol.

3. The method of claim 1, further including treating with a selected dose of 20S+ pregnanolone or 22R+ pregnanolone or a combination thereof.

4. The method of claim 2, further including treating with a selected dose of troglitazone.

5. The method of stimulating markers of osteoblastic differentiation in cells with a selected dose of at least one compound selected from the family of oxysterols, precursors or derivatives thereof.

6. The method of claim 5 wherein the marker is alkaline phosphatase activity or calcium incorporation.

7. The method of claim 5 wherein the oxysterol is any one or combination of 20S-hydroxycholesterol, 22S-hydroxycholesterol and/or 25-hydroxycholesterol.

8. The method of claim 7, further including treating with a selected dose of 20S+ pregnanolone or 22R+ pregnanolone or a combination thereof.

9. The method of claim 8, further including treating with a selected dose of troglitazone.

10. The method of claim 5 wherein the cells are selected from the group comprising marrow stromal cells, stem cells, osteoprogenitor cells or calvarial organ cultures.

COMPOUNDS AND METHODS FOR ENHANCING BONE FORMATION

ABSTRACT OF THE DISCLOSURE

Osteoporosis is still a major cause of morbidity and mortality and is mainly treated by agents that interfere with osteoclastic bone resorption. However, osteoporosis also involves decreased bone formation as a result of reduced osteoblastic cell number and bone forming activity. The mechanism(s) underlying these changes in cellular profile and function in osteoporosis is not clear. We recently reported that the cholesterol biosynthetic pathway of marrow stromal cells (MSC) is important in their osteoblastic differentiation, as demonstrated by the inhibitory effects of HMG-CoA reductase inhibitors, which could be reversed by mevalonate. In the present study we identify several oxysterols as possible products of the cholesterol biosynthetic pathway that are important in mediating osteoblastic differentiation of MSC. Oxysterols are formed by oxygenation of cholesterol in part through the actions of cytochrome P450 enzymes. M2-10B4 (M2) mouse marrow stromal cells undergo osteoblastic differentiation in an osteogenic medium containing fetal bovine serum, ascorbate and B-glycerophosphate. Treatment of M2 cells with the oxysterols 22(R)-, 22(S)-, and 20(S)-hydroxycholesterols (1-10 μM) significantly enhanced alkaline phosphatase activity, an early marker of osteoblastic differentiation, as early as 2 days post-treatment, and with relative potencies: 20(S)>22(S)>22(R). This enhancement was between 2-10 fold and was persistent up to 12 days. After 10 days of treatment with the oxysterols, mineralization, a late marker of osteoblastic differentiation, was also enhanced 2-5 fold. In contrast, 7-ketocholesterol (1-10 μM) did not have these effects. Furthermore, two other products of the cholesterol biosynthetic pathway, farnesol and geranylgeraniol had no effect or an inhibitory effect, respectively, on markers of osteoblastic differentiation. Osteoporosis is also characterized by increased marrow adipocytes at the expense of osteoblastic cells, and marrow stromal cells undergo adipogenic differentiation in the presence of PPAR-γ agonist troglitazone. Interestingly, the osteoinductive oxysterols also inhibited troglitazone-induced adipogenesis of M2 cells. Oxysterol-mediated stimulation of osteoblastic bone formation and inhibition of adipogenesis of progenitor cells are potential new strategies for prevention and treatment of osteoporosis.

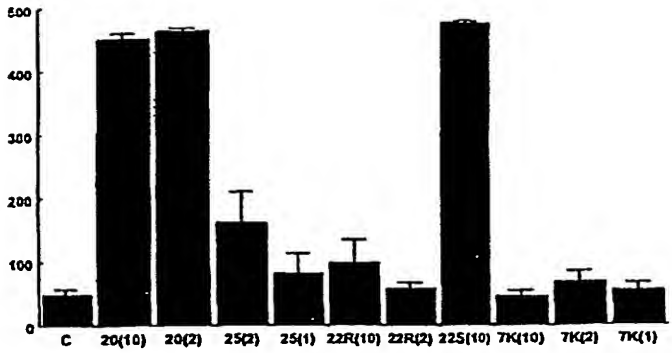

Figure 1. Effect of oxysterols on marrow stromal cells. M2-10B4 cells were treated at 90% confluence with vehicle (C) with one of the following oxysterols at the concentration indicated (in μM): 20S-Hydroxycholesterol, 25-Hydroxycholesterol, 22R-Hydroxycholesterol; 22S-Hydroxycholesterol; 7-ketocholesterol. After 4 days, alkaline phosphatase activity was determined in whole cell lysates and normalized to protein.

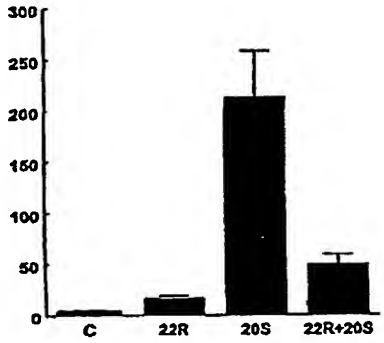

Figure 2. Effect of 24 hours treatment of marrow stromal cells with oxysterols. M2 cells were treated at 90% confluence with vehicle (C), or oxysterols 22R-Hydroxycholeterol or 20S-Hydroxycholesterol, each at 5 μM, alone or in combination. After 24 hours, the cells were rinsed and media replaced with out oxysterols. After 4 days, alkaline phosphatase activity was measured in whole cell extracts and normalized to protein.

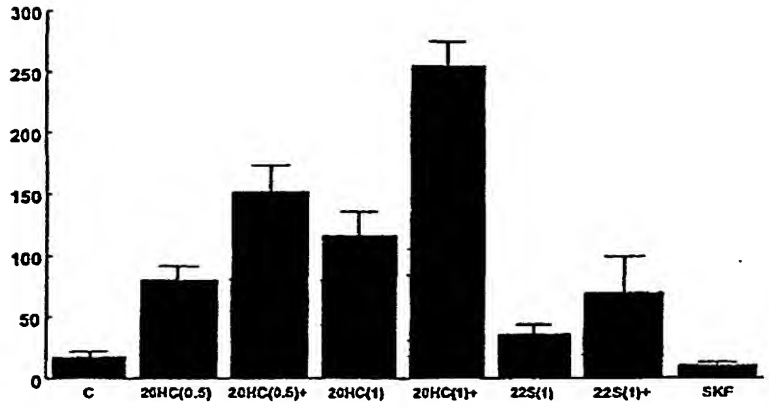

Figure 3. Effect of oxysterols and cytochrome P450 inhibitor SKF525A on marrow stromal cells. M2 cells were treated at 90% confluence with vehicle (C), or oxysterols 20S-Hydroxycholesterol (20HC) or 22S-Hydroxycholesterol at (0.5 μM) or (1 μM), in the absence or presence of SKF525A 10 μM (+). After 4 days, alkaline phosphatase activity was measured in whole cell extracts and normalized to protein.

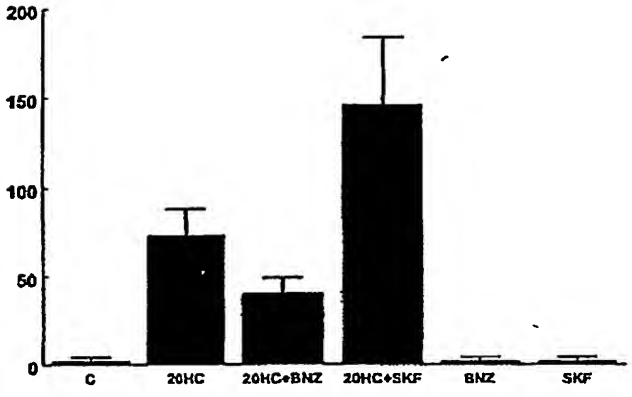

Figure 4. Effect of oxysterols and cytochrome P450 activator Benzylimidazole and inhibitor SKF525A on marrow stromal cells. M2 cells were treated at 90% confluence with vehicle (C), or 20S-Hydroxycholesterol (20HC) 2 μM, in the absence or presence of Benzylimidazole 50 μM or SKF 10 μM. After 4 days, alkaline phosphatase activity was measured in whole cell extracts and normalized to protein.

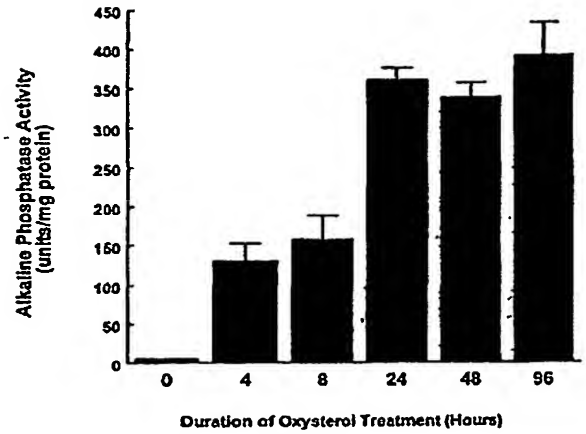

Figure 5. Effect of duration of treatment with combination oxysterols on marrow stromal cells. M2 cells were treated at 90% confluence with the combination of 22R-Hydroxycholesterol and 20S-Hydroxycholesterol, each at 5 μM, for 4 to 96 hours. The oxysterols where removed and fresh media without oxysterols was added for a total duration of 96 hours. Alkaline phosphatase activity was measured in whole cell extracts and normalized to protein.

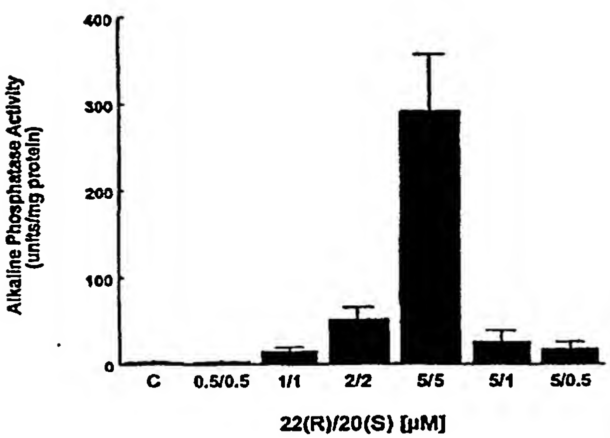

Figure 6. Effect of combination oxysterols on marrow stromal cells. M2 cells were treated as previously described with the combination doses of 22R-and 20S-Hydroxycholesterol. After 4 days, alkaline phosphatase activity was measured in whole cell extracts and normalized to protein.

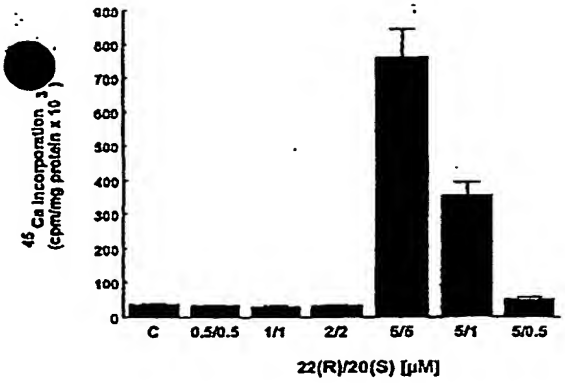

Figure 7. Effect of combination oxysterols on marrow stromal cells. M2 cells were treated as previously described with the combination doses of 22R-and 20S-Hydroxycholesterol. After 10 days, 45Ca incorporation was measured to assess bone mineral formation, and normalized to protein.

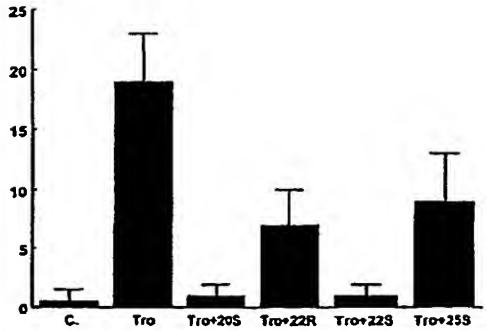

Figure 8. Effect of oxysterols on adipogenesis of marrow stromal cells. M2 cells at confluence were treated with vehicle (C), troglitazone 10 μM (Tro), alone or in combination with 10 μM oxysterols 20S-, 22R-, or 25S-hydroxycholesterol. After 8 days, adipocytes were identified by oil Red O staining and quantified by counting under a phase contrast microscope.

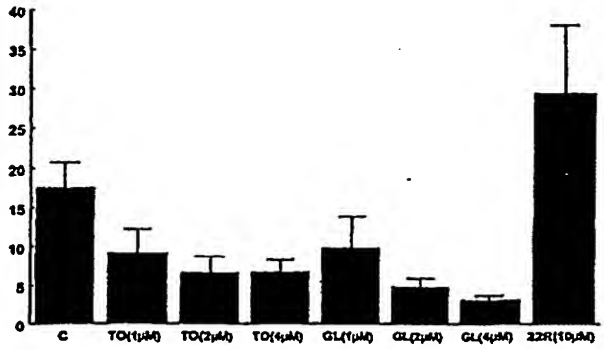

Figure 9. Effect of LXR activators on marrow stromal cells. M2 cells at 90% confluence were treated with vehicle (C), or two unrelated LXR ligands, TO and GL, at 1-4 μM, or 22R-hydroxycholesterol at 10 μM. After 4 days, alkaline phosphatase activity was measured in whole cell lysates and normalized to protein.